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Proteomic profiling of *Rhipicephalus* (*Boophilus*) *microplus* midgut responses to infection with *Babesia bovis*

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Abstract

Differences in protein expression in midgut tissue of uninfected and *Babesia bovis*-infected southern cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*, were investigated in an effort to establish a proteome database containing proteins involved in successful pathogen transmission. The electrophoretic separation of midgut membrane proteins was greatly improved by using liquid-phase isoelectric focusing combined with one-dimensional or two-dimensional (2-D) gel electrophoresis. A selection of differentially expressed proteins were subjected to analysis by capillary-HPLC-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS). Among the identified *Babesia*-affected tick midgut proteins were six proteins that are implicated in signaling processes, including three Ca^{2+}-binding proteins, a guanine nucleotide-binding protein, a protein with signal peptide activity and a translocon-associated receptor protein. Up-regulation of five metabolic enzymes indicated parasite-induced changes in electron and proton transport, protein processing and retinoic acid metabolism. Among the down-regulated proteins were a molecular chaperone, a cytoskeletal protein and a multifunctional protein of the prohibitin family. Identification of these proteins may provide new insights into the molecular interactions between *B. bovis* and its tick vector, and could lead to identification of anti-tick and transmission-blocking vaccine candidates.
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1. Introduction

The southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, was declared to be eradicated from the US by 1943 (Graham and Hourrigan, 1977), and all cattle presented for importation at the Texas-Mexico border are treated with acaricides to prevent reintroduction. However, there is a risk of the reestablishment of this tick in the US due to the presence of acaricide-resistant populations in Mexico (Miller et al., 2005). *Rhipicephalus microplus* is a vector for the protozoan agents of cattle fever, *Babesia bovis* and *Babesia bigemina*. Since cattle fever is endemic in Mexico, and all imported cattle are checked only for ticks but not for infection with this parasite, there is a constant threat of the resumption of enzootic transmission of cattle fever in the US. This threat has triggered the search for new, environmentally safe and effective techniques for control of *R. microplus* that can be integrated with conventional chemical control methods.

Anti-tick vaccines are a potential alternative to chemical control methods, but so far the small number of tick-protective antigens that have been identified has

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been a limiting step in vaccine development (de la Fuente and Kocan, 2006). It is obvious, that developing novel tick control strategies will require better knowledge of the complement of proteins expressed in *R. microplus*. Two proteome studies have been published for *R. microplus* to date. We have access to sequence information for 20 abundantly expressed larval proteins, representing multiple cuticular proteins, a cytoskeletal protein, a salivary gland-associated protein, several housekeeping proteins, and tropomyosin (Untalan et al., 2005). Recently, we identified 19 membrane proteins that were differentially expressed in the ovaries in uninfected and *Babesia*-infected ticks. Among the ovarian proteins that were up-regulated in infected ticks were calreticulin, two myosin subunits, an endoplasmic reticulum protein, a peptidyl-prolyl *cis-trans* isomerase (PPIase), a cytochrome c oxidase subunit, a glutamine synthetase, and a family of Kunitz-type serine protease inhibitors. Among the down-regulated ovarian proteins were another PPIase, a hemoglobin subunit, and a lysozyme (Rachinsky et al., 2007). The current study describes an extensive survey of proteins that are regulated in response to infection and is part of an ongoing effort to establish a proteome database that can be utilized to identify specific proteins that may be involved in successful pathogen transmission.

Tick midgut proteins are considered to be concealed antigens since they are normally hidden from the bovine host's immune system (Nuttall et al., 2006). A potential pitfall of concealed antigens is that they are not boosted by tick feeding and may require several immunizations of the host in order to increase vaccine-induced antibodies to levels that are high enough to cause an anti-tick effect. Components of tick saliva are commonly investigated as anti-tick vaccine candidates, however, these exposed antigens may have the disadvantage of having immunomodulatory effects (Nuttall et al., 2006). The use of concealed gut antigens for vaccine development may circumvent this problem. Midgut antigens are particularly promising targets for the development of vaccine strategies, because antigens on the lumenal surface of the midgut are directly exposed to the blood meal and host immune effectors contained in it. Since blood meal digestion in ticks is intracellular (Sonenshine, 1991), host immunoglobulins may also contact intracellular midgut antigens. Midgut antigens of *R. microplus* have previously been exploited as targets for vaccine development. For example, the midgut protein Bm86, which has been shown to reduce survival and reproduction rates of ticks, has been the basis for a commercial vaccine (Willadsen, 2004). Glycosylation of tick proteins appears to be important for enhancing the protective capacity of tick vaccine antigen preparations, as demonstrated in studies with yeast-derived recombinant Bm86 and Bm95 proteins (reviewed in de la Fuente et al., 2006a). Minor differences in the nucleotide sequences of homologous Bm86 genes isolated from different tick strains can have an effect on how efficiently an antigen protects cattle. It was shown that a Bm95-based vaccine was able to protect cattle against infections with both Bm86-sensitive and Bm86-resistant *R. microplus* strains. This suggests that the Bm95 homologue of Bm86 could yield a more universal vaccine, which might be able to protect cattle against *R. microplus* strains from different geographical regions (Garcia-Garcia et al., 2000).

Serine proteinases isolated from tick midguts may also be promising candidate tick vaccine antigens. It was shown that three serine proteinases from *Rhipicephalus appendiculatus* were differentially expressed in relation to events relating to blood meal uptake or digestion in ticks. This raises the possibility that a vaccine based on these proteins could interfere with the ability of the tick to feed successfully (Mulenga et al., 2003). Another potential anti-tick vaccine candidate is a 35 kDa midgut antigen isolated from the three-host tick *Rhipicephalus haemaphysaloides*. This antigen induced protection against tick feeding when it was used to immunize rabbits (Kavitha et al., 2007).

Host immunoglobulins are taken up with a blood meal and retain biological activity while exposed to midgut tissue and during their passage into the hemolymph and various tissues of the ticks (da Silva Vaz et al., 1996; Wang and Nuttall, 1999). The midgut is also an important site for development of the sexual stages of *Babesia* parasites that are taken up with a blood meal. In the midgut lumen, *Babesia* parasites undergo a series of developmental changes. After ingestion with the blood meal, the parasites differentiate into micro and macro gametes; gametes fuse to form zygotes which then develop into mobile kinetes. The kinetes selectively invade digestive cells of the tick gut, where they multiply. Kinetes enter the hemolymph after leaving gut cells and invade other tick tissues, i.e. the ovaries in the case of *B. bovis* (Bock et al., 2004). Vaccines that are based on tick midgut antigens could, therefore, serve a dual purpose: they could control ticks by attacking their digestive system and making them unable to successfully digest the blood meal, or they could interfere with parasite sexual development by disrupting gut homeostasis, thus preventing pathogen development and transmission. The identification of appropriate antigenic targets in the tick gut is the main

difficulty in accomplishing this objective. Thus, the goal of this study was to increase our knowledge about potential antigenic targets in the *R. microplus* midgut, with particular emphasis on membrane proteins that are differentially expressed in response to infection with *B. bovis*. We targeted the adult midgut because it contains developmental forms of *B. bovis* (Riek, 1966) and is the site of critical parasite life cycle events: gametogenesis, syngamy and subsequent zygote and kinete formation. It is reasonable to assume that there would be tissue-specific gene expression in response to these events. This approach may provide us with antigenic targets that could be combined to develop broad-spectrum vaccines that affect both the tick and the pathogens transmitted by it.

2. Material and methods

2.1. Ticks

The *R. microplus* ticks used in these studies were from the La Minita strain maintained at The University of Idaho Holm Research Center (HRC) since 1999; at the time of these studies this colony was in approximately the 20th laboratory generation. Ticks for this colony were originally collected in Starr Co. Texas in 1996. Holstein calves 5–6 months of age were used in these experiments. All cattle were cared for in facilities located at the HRC following procedures approved by the University of Idaho Institutional Animal Care and Use Committee. We used uninfected and *B. bovis*-infected female ticks to collect midguts and ovaries, which in addition to the study described here were used for an analysis of infection-induced changes in the ovary proteome (Rachinsky et al., 2007) and microarray studies characterizing infection-induced changes in gene expression (Guerrero et al., unpublished results).

To obtain uninfected ticks, *R. microplus* larvae from 1.0 g of eggs were placed on a calf. On day 22 the first replete tick females were collected and they continued to drop until the animal was euthanized on day 31. Replete female ticks for this study were collected on day 22 and dissections were done 2 days post repletion while females were actively ovipositing.

To obtain infected ticks, *R. microplus* larvae from 1.0 g of eggs were placed on a calf on 2 days (day 1 and again on day 3; total larvae from 2 g of eggs) to ensure that replete females that had fed through the period of peak parasitemia could be obtained. The calf was artificially infected with *B. bovis* by inoculating it intravenously with 1.8 ml of *B. bovis* blood stabilate (T2Bo strain of *B. bovis*, made 14 November 1989 at 5.5% parasitized erythrocytes and stored in liquid nitrogen since it was made) on day 14. The calf reached its peak level of infection as determined by rectal temperature on approximately day 22. The first replete females were collected on day 20, and they continued to drop until the animal was euthanized on day 24. Replete female ticks for this study were collected on day 24, and dissected 4 days post repletion. Only apparently healthy, actively ovipositing tick females were dissected (Howell et al., 2007). To confirm infection of ticks with *B. bovis*, hemolymph smears were examined from 66 females collected on this day: 46 females had $\geq$5 kinetes per high power field; the remaining 20 females had $\geq$3 kinetes per high power field.

2.2. Tissue dissection and protein extraction

Midguts were dissected from engorged adult females, that were either infected with *B. bovis* (infected ticks) or not (control ticks). Ticks were dissected under phosphate buffered saline (PBS). The ventral cuticle was excised with a scalpel, the midgut was removed, rinsed in sterile PBS then held in PBS on ice in groups of 5 ticks per tube until they were frozen at −80 °C. Protein isolation was performed as described earlier (Rachinsky et al., 2007), using the ReadyPrep Sequential Extraction Kit (BioRad, Hercules, CA). Protein extractions were done in duplicate, using midgut tissue from a total of 20 control ticks and 20 infected ticks.

To midgut tissue from 10 individual ticks, 0.4 ml Reagent 1 with 10 μl protease inhibitor cocktail (Protease Arrest, GenoTech, St. Louis, MO) was added. Midgut tissue was homogenized for 3 × 30 s on ice, using disposable pellet pestles. After adding another 2.6 ml Reagent 1 with 68 μl protease inhibitor cocktail, the midgut tissue was further homogenized using three 10 s bursts with a Polytron. To break down nucleic acids, 30 μl nuclease (FOCUS Nuclease, GenoTech, St. Louis, MO) was added. Midgut extracts were incubated for 30 min at room temperature, and then ultracentrifuged at 200,000 × *g* for 2 h at 4 °C. The supernatant, which mainly consists of Tris-soluble cytosolic proteins, was aliquoted and stored at −80 °C (fraction A). To minimize carry-over of Tris-soluble proteins into subsequently extracted fractions, pellets were resuspended twice in 1 ml Reagent 1 containing protease inhibitor, and re-centrifuged at 200,000 × *g* for 2 h at 4 °C; supernatants were discarded. The remaining pellets were resuspended in 1 ml Reagent 2 containing 2 mM of the reducing agent tributylphosphine (TBP), incubated for 1 h at 100 rpm on an orbital shaker at room temperature, and then

ultracentrifuged at 200,000 × *g* for 2 h at 4 °C. The supernatant, containing urea-soluble membrane and membrane-associated proteins, was aliquoted and stored at −80 °C (fraction B).

Fractions A and B were analyzed by one- and/or two-dimensional gel electrophoresis. Protein concentrations were determined using a protein assay (BioRad) based on the Bradford dye-binding procedure (Bradford, 1976), which was modified to allow protein determination in samples with high concentrations of detergents, according to the instructions provided by the manufacturer.

2.3. One-dimensional (1-D) gel electrophoresis

Aliquots containing 2 μg each of protein fractions A and B from midguts of control and infected ticks were compared using one-dimensional electrophoresis on precast SDS-polyacrylamide gradient gels (NuPage 4–12% Bis-Tris Gels; Invitrogen, Carlsbad, CA). Electrophoresis, staining and imaging of gels were performed as described in Rachinsky et al. (2007).

2.4. Isoelectric focusing (IEF) and second dimension gradient gel electrophoresis

Sample fractionation was performed in a liquid-phase IEF system using the MicroRotofor Liquid-Phase IEF Cell (BioRad), followed by a second IEF step of selected pooled fractions on IPG strips of the appropriate pH range. This method was applied to remove proteins that are outside the pH range of interest and, thereby, improve the resolution and detection of low-abundance proteins (Harbers et al., 2006). Midgut proteins from either control ticks or infected ticks were fractionated in the MicroRotofor using equal total amounts of protein and the same focusing conditions. A sample containing a total of 3 mg protein was first reduced and alkylated using TBP and iodoacetamide, following the protocol for the ReadyPrep reduction-alkylation kit (BioRad). For fractionation in the MicroRotofor cell, the sample was then diluted to 2.7 ml in an IEF buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2 mM TBP, 0.001% bromophenol blue, and 2% (w/v) Bio-Lyte 3/10 ampholytes (Harbers et al., 2006). The sample was loaded into the focusing chamber of the MicroRotofor cell and focused according to the manufacturer's instructions at 1 W (constant) for a total of 4 h. All ten fractions were collected. Fraction volumes were calculated by weight/density (density = 1.1 g/ml; Harbers et al., 2006) and protein concentration was determined using a modified BioRad protein assay as described above. The pH of each fraction was estimated using the Fisher Alkacid Full Range pH Kit (Fischer Scientific, Pittsburgh, PA).

Equal aliquots of respective fractions from control and infected ticks were screened for major differences in protein patterns on polyacrylamide gradient gels. In a first set of experiments, equal volumes of MicroRotofor fractions 1–10 were applied directly to precast SDS-polyacrylamide gradient gels (NuPage 4–12% Bis-Tris Gels; Invitrogen) and separated as described above. In a second set of experiments, selected liquid-phase IEF fractions were pooled and subjected to an additional IEF step on narrow pH range IPG strips. Equal volumes of fractions 1–5, containing a total amount of 100 μg protein, were treated with the ReadyPrep 2-D cleanup kit (BioRad), then resuspended in rehydration buffer and applied to pH 3–6 IPG strips (BioRad). Equal volumes of fractions 6–7 and 8–10 (100 μg total protein each) were treated accordingly and applied to pH 5–8 or 7–10 IPG strips. IEF on IPG strips was performed in a PROTEAN IEF Cell (BioRad) using the focusing conditions recommended by the manufacturer. Equilibration of focused IPG strips and second dimension gradient gel electrophoresis on precast 4–20% Tris–HCl Ready gels (BioRad) was performed as described in Rachinsky et al. (2007). Separated proteins on both types of gels were stained with Sypro Ruby Protein Gel Stain (Invitrogen) using the Rapid Protocol for staining as described by the manufacturer. Imaging of gels and analysis of protein spot patterns were performed as described earlier (Rachinsky et al., 2007). Selected differentially expressed proteins were subjected to further analysis by mass spectrometry.

2.5. Spot excision and sample preparation for mass spectrometry

Proteins selected for further analysis by mass spectrometry were excised either from 1-D gels of liquid-phase IEF fractions, or from 2-D gels of protein samples that had been subjected to dual IEF (liquid-phase IEF plus IEF of selected fractions on IPG strips, as described above). Dual IEF greatly increased the resolution of low abundance proteins and allowed us to choose from a broader range of differentially expressed proteins. Gel spots of interest were excised and collected in microtiter plates using a ProteomeWorks Spot Cutter controlled by PDQuest Basic Excision Software (BioRad). Only those protein spots that showed consistent expression differences between control and infected tissues in at least two independently performed electrophoresis runs were considered

as differentially expressed. Destaining and dehydrating of gel plugs and in-gel digestion of proteins were performed as previously described (Rachinsky et al., 2007).

2.6. Mass spectrometry and database search

Capillary-HPLC-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) was performed on a Thermo Fisher LTQ linear ion trap mass spectrometer coupled to an Eksigent NanoLC micro HPLC by means of PicoView (New Objective; Boston, MA) nanospray interface. Capillary on-line HPLC separation of tryptic peptides was conducted using the following conditions: column, New Objective PicoFrit (75 μm i.d.) packed to 10 cm with C18 adsorbent (Vydac 218MSB5); mobile phase A, 0.5% acetic acid/0.005% TFA in distilled water (Honeywell Burdick & Jackson; Morristown, NJ); mobile phase B, 90% ACN/0.5% acetic acid/0.005% TFA in distilled water; gradient, 2–42% B in 30 min; flow rate, 0.4 μl/min. A data-dependent acquisition protocol was employed in which one survey scan was acquired followed by seven collision-induced dissociation (CID) spectra. Mascot (Matrix Science; London, England) was used to search the CID spectra against the SwissProt database (http://www.expasy.org/sprot/) and the *Boophilus microplus* Gene Index (BmiGI) (http://www.compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/tc_ann.pl?gudb=b_microplus) translated with prot4EST (Wasmuth and Blaxter, 2004). Variable peptide modifications considered included methionine oxidation and cysteine carbamidomethylation. Only peptide spectra with a probability of peptide ID greater than or equal to 95% were considered. We used Scaffold (Proteome Software; Portland, OR) to validate and organize mass spectrometry data and to cross-correlate Mascot search results with X! Tandem search results, resulting in reliable protein identification (Scaffold data available on request). For the above-mentioned BmiGI database a total of 42,512 ESTs were used, which assembled into 9403 tentative consensus (TC) sequences and 4240 singletons (Guerrero et al., 2007). Protein identity was determined by submitting TC sequences that matched to differentially expressed tick midgut proteins to protein database searches using the NCBI/BLAST/blastp suite at http://www.ncbi.nlm.nih.gov/blast/. The protein ID labels we used are UniProt or GenBank accession numbers. The Gene Ontology Database (http://www.geneontology.org/) and databases at UniProt (http://www.pir.uniprot.org/), Pfam (http://www.sanger.ac.uk/Software/Pfam), InterPro (http://www.ebi.ac.uk/interpro) were mined for information about protein classification, in particular for information about molecular and biological functions of identified proteins.

3. Results

3.1. Comparative 1-D analysis of tick midgut proteins

The majority of extracted midgut proteins were Tris-soluble. In control ticks, 97.3% of the total amount of fractionated protein was collected in fraction A. Membrane or membrane-associated proteins collected in fraction B amounted only to 2.7%. In infected ticks, 96.4% of the total amount of fractionated protein was retrieved in fraction A, 3.6% in fraction B.

Comparison of protein fractions obtained from control and infected ticks on 1-D gels showed a few

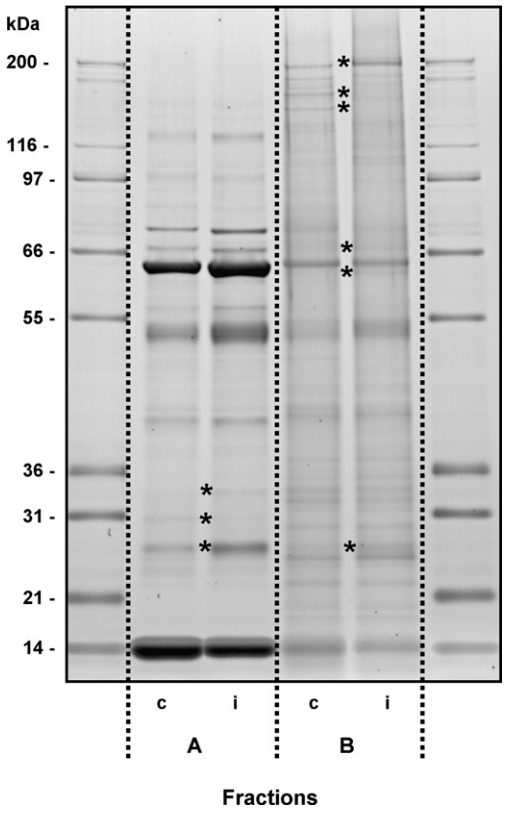

Fig. 1. Comparison of Tris-soluble cytosolic (fraction A) and membrane and membrane-associated (fraction B) midgut proteins from uninfected control ticks (c) and *Babesia*-infected ticks (i). Fractions A and B were extracted using reagents with increasing solubilizing capacities (see text for details). One-dimensional separation was performed on 4–12% gradient polyacrylamide gels. Molecular masses in kDa are indicated on the left. Asterisks indicate differences in midgut protein expression between control and infected ticks.

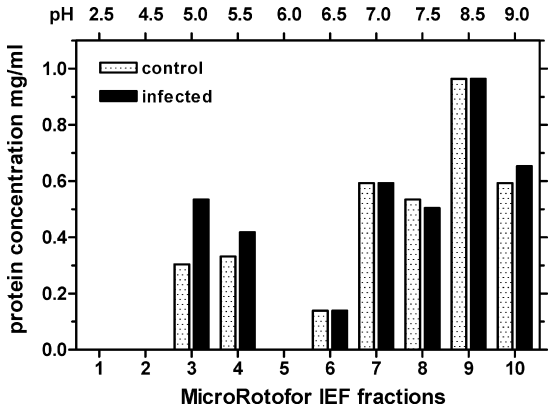

Fig. 2. Protein distribution in fractions obtained by liquid-phase IEF in a MicroRotofor cell, shown for membrane proteins that were extracted from midguts of uninfected control ticks (dotted bars) and *Babesia*-infected ticks (solid black bars). The pH gradient ranged from approx. 2.5 in fraction 1 to approx. 9.0 in fraction 10.

subtle differences in low abundance Tris-soluble proteins (Fig. 1; fraction A). There appeared to be a few differences between control and infected ticks among the membrane-associated proteins contained in fraction B, although these differences were also subtle (Fig. 1). The poor separation of fraction B membrane proteins on 1D gels and high levels of background staining led us to utilize additional purification and separation techniques to better resolve infection-induced changes in protein expression. We used two different isoelectric focusing techniques followed by separation of proteins by mass in the second dimension on gradient polyacrylamide gels to achieve satisfactory resolution of these midgut proteins.

3.2. Comparative 2D-analysis of membrane proteins from tick midguts

Adding liquid-phase isoelectric focusing to the protocol greatly increased the relative concentration and resolution of low-abundance gut membrane proteins. Proteins were collected in ten fractions with pH values ranging from 2.5 to 9.0. Protein concentrations were highest in the basic pH range fractions 7–10 (Fig. 2). Protein concentrations appeared to be higher for infected tick midguts in fractions 3 and 4 (pH range 5–6). This might have been caused by an increase in the expression of several highly abundant proteins (Fig. 3; protein bands B2, B3, B4, B6, and B7).

Fig. 3 shows the results of a side-by-side-comparison of protein patterns in respective liquid-phase IEF fractions from control and infected ticks. Equal volumes of each fraction were loaded and, therefore, the protein patterns reflected the unequal distribution of proteins within the pH range, with the majority of proteins shifted towards the basic end of the pH range. Twenty differentially expressed protein bands were submitted to MS/MS analysis. Sixteen protein bands (Fig. 3) yielded reliable (≥95% probability) peptide spectra, matching to a total of nineteen ESTs in the *R. microplus* translated EST database (Fig. 3; Table 1). Sixteen of these ESTs yielded a protein ID based on matches to proteins in databases available through NCBI/BLAST/blastp (Table 1). Protein band B16 yielded peptides that matched two translated ESTs, TC5777 and TC9698, which both matched the same *Ixodes scapularis* putative saliva secreted protein (accession no. Q4PN29). When compared to each other, the translated

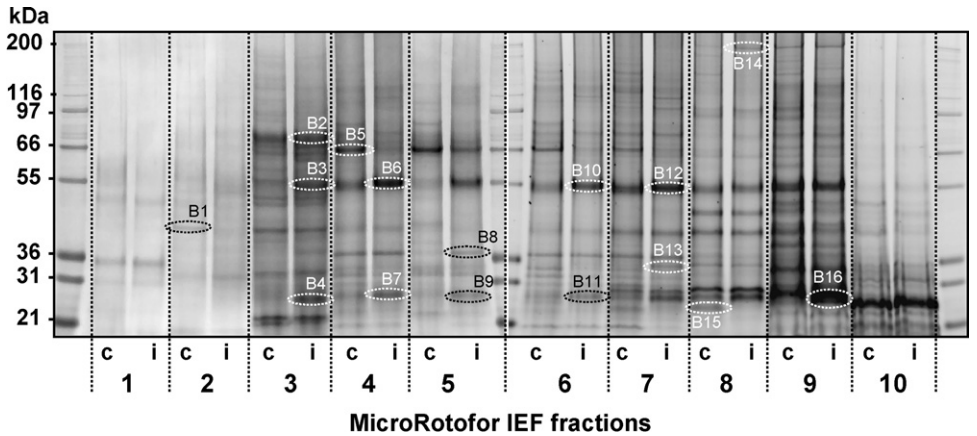

Fig. 3. Composition of midgut membrane proteins in respective MicroRotofor fractions 1–10 from uninfected control ticks (c) and *Babesia*-infected ticks (i). The circled differentially expressed proteins B1 to B16 were analyzed by MS/MS, and the results are shown in Table 1. Equal volumes of each fraction were analyzed on 4–12% gradient polyacrylamide gels. Molecular masses in kDa are indicated on the left. The pH gradient ranged from approx. 2.5 in fraction 1 to approx. 9.0 in fraction 10.

Table 1
Comparison of MicroRotofor fractions: *R. microplus* midgut proteins up/down-regulated in *Babesia*-infected females

Spot #	Up/ down-regulation	Match to *R. microplus* translated EST[a]	Peptide sequences[b]	% Coverage[c]	Mr (kDa)[d] theoretical/ observed	pI[e] theoretical/ observed	EST match to accession no.[f]	*E* value	Protein ID [f]
B2	(+)	TC7442	FASEVAGVDDLGTTGR; QELLVADDNAK; FTDVQYSGK; AVEDKDVGPLVK	15	35.3/∼70	6.35/∼5.0	Q7PXY3	1e−98	ENSANGP00000022170 [*Anopheles gambiae* str. PEST]
B3	(+)	TC9513	EVTLSEYMK; DNGVDILVPK; ELATAINNLFK; FSSLHYSIENAENNEMAAK	18	29.9/∼55	5.90/∼5.0	–	–	n.i.[g]
B4	(+)	TC8983	SEDGGVLANVDFR; ITSGTALVLFR; VRPEVEESAFQGITR; TLYLEETIK	10	53.8/∼27	5.60/∼5.0	P07314	2e−105	Gamma-glutamyltransferase 1 [*Rattus norvegicus*]
B6	(+)	TC9513	EVTLSEYMK; DNGVDILVPK; ELATAINNLFK	11	29.9/∼55	5.90/∼5.5	–	–	n.i.[g]
B6	(+)	TC14596	VLSIGDGIAR; VLGQATTANLEETGR; AVDSLVPIGR; VVDALGNPIDGK	15	33.8/∼55	9.23/∼5.5	Q000T7	2e−137	Mitochondrial H+ ATPase a subunit [*Pinctada fucata*]
B7	(+)	TC6480	EVDQEEKDIDEYR; QmSVYEEVK; LDYHLEVQNVTR; LGGIEAYK;	16	30.7/∼28	7.79/∼5.5	Q4PM57	6e−106	Putative ATP synthase-like protein [*Ixodes scapularis*]
B7	(+)	TC8929	RGDNALLDYK; GDNALLDYK; YQSEVELQTIK; NVQDFSTAR;	17	20.3/∼28	7.75/∼5.5	Q4PLZ7	1e−88	Probable microsomal signal peptidase 22 kDa subunit [*Ixodes scapularis*]
B8	(+)	TC9038	EDLTEVR; GNVGFVFTK; SNYFLR; FAAAAAPAAGGGAAAAKPEESKK; GHLDNNPALEK; SAFVEGVR; EYLKDPSK; HAVLLMGK; LVQLLDEYPK;	28	34.8/∼38	5.87/6.0	Q4PMB4	2e−144	60S acidic ribosomal protein P0 [*Ixodes scapularis*]
B9	(+)	–	DLTDYLMK; GYSFTTTAER; EITALAPSTMK	8	41.7/∼30	5.3/∼6.0	Q6X4V4	n/a	Actin [*Boophilus microplus*]
B10	(+)	TC14596	VLSIGDGIAR; VLGQATTANLEETGR; AVDSLVPIGR; VVDALGNPIDGK; APGIIPR; STVAQIVK;	19	33.8/∼55	9.23/∼6.5	Q000T7	2e−137	Mitochondrial H+ ATPase a subunit [*Pinctada fucata*]
B10	(+)	TC5764	YAQEDIDTSVK; VSFTGSTEVGK; ILGLIESGK; VNLELGGK;	8	46.2/∼55	5.79/∼6.5	P27463	2e−139	Aldehyde dehydrogenase 1A1 [*Gallus gallus*]
B11	(+)	TC8929	RGDNALLDYK; GDNALLDYK; YQSEVELQTIK; NVQDFSTAR;	17	20.3/∼28	7.75/∼6.5	Q4PLZ7	1e−88	Probable microsomal signal peptidase 22 kDa subunit [*Ixodes scapularis*]
B12	(+)	TC9694	ELIIGDR; VLSIGDGIAR; TAIAIDGIINQK; AVDSLVPIGR; VVDALGNPIDGK; STVAQIVK;	11	58.1/∼55	8.59/∼7.0	Q08BA1	0.0	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle [*Danio rerio*]
B12	(+)	TC5764	LADLLER;VSFTGSTEVGK; ILGLIESGK; VNLELGGK;	8	46.2/∼55	5.79/∼7.0	P27463	2e−139	Aldehyde dehydrogenase 1A1 [*Gallus gallus*]
B13	(+)	TC12042	(R)DILAVSIER(T); (K)FQPSTITGLGTIQR(S);	14	24.6/∼34	9.01/∼7.0	–	–	n.i.[g]
B13	(+)	TC6908	FPETVLSASR; DETSYGIPK; SLVDELRPEVmTTNAK;	11	36.1/∼34	7.09/∼7.0	Q4PM32	0.0	Guanine nucleotide-binding protein [*Ixodes scapularis*]
B14	(+)	TC12227	IILPEGATDIK; LKQESIEAAEPVNELQR; LQVSGLLEK; DASGFQASLK;	18	30.3/∼200	7.92/∼7.5	XP_782614	2e−56	Predicted: similar to Ribophorin I [*Strongylocentrotus purpuratus*]
B16	(+)	TC5777	TNAFVEATAPFGQQATLK; TLTVSGIEFR; AQFPSGmLYGLSSVVR; qDANNYIDTVLR; QSASNILLGIVNSSFR; DHLPANVR; SLNLDPTHLPGFNFK; VPLPSP;	46	23.5/∼28	9.73/∼8.5	Q4PN29	3e−20	Putative salivary secreted protein [*Ixodes scapularis*]
B16	(+)	TC9698	TLSLNSLELK; SVPAVLK; VDFTSGLVR; EALNQAVR; LGLNNER; DRLPGEVR;	22	24.1/∼28	9.59/∼8.5	Q4PN29	2e−16	Putative salivary secreted protein [*Ixodes scapularis*]
B16	(+)	TC9611	SYEQEFAR; DLPLYQVIEK; YIDQVLGYQmADLVR;	24	23.2/∼28	9.33/∼8.5	–	–	n.i.[g]
B1	(−)	TC12726	LTPEDIER, FDLTGIPPAPR, YLEQNPDADAEQLK, IVISNDQNR, SLEEIVQPIVAK	25	24.0/∼42	4.87/∼4.5	Q0MUU6	2e−82	Heat shock cognate 70 protein (*Trichoplusia ni*)
B5	(−)	TC7873	QTEITEIDGR; DLEQLLEQER; TALLSSEGEEVATR; VRDLEQLLEQER; LADTLQELR; IAELESLNQSLSSR; TLNEQLDFQK; SSGIGGASGFR; DLQELLDR; KLADTLQELR; EYQDLLDIK;	26	43.9/∼66	6.03/∼5.5	XP_396670	2e−85	Predicted: similar to Lamin CG6944-PA, partial [*Apis mellifera*]

B15	(–)	TC13955	NVLSAEYVR; LILDVK; AVALVDELFR; VPIITDTLR;	10	39.0/~24	6.32/~7.5	Q7ZUV3	1e−62	Hypothetical protein LOC393213 [*Danio rerio*]
B15	(–)	TC6772	TQDNSVIIHGK; NFTPEEITVK; NFTPEEITVK;	16	21.2/~24	7.86/~7.5	Q86G69	3e−69	Putative heat shock-related protein [*Dermacentor variabilis*]
B15	(–)	–	ELVDDSINNVR; VQLVVGDGR; VFEVmLATDR; SGGASHSELIHNLR;	19	24.5/~24	6.78/~7.5	P15246	n/a	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase

[a] Translated EST from a *Rhipicephalus* (*Boophilus*) *microplus* EST database (Guerrero et al., 2007) to which significant peptide sequence similarity was found.
[b] Sequence information obtained from capillary-HPLC-electrospray tandem mass spectrometry. Probabilities of peptide ID were ≥95%.
[c] % Coverage of the respective EST provided by the identified peptide sequences.
[d] Theoretical molecular mass of translated *R. microplus* EST (using compute pI/Mw tool on http://www.expasy.org)/observed mass estimated from position of protein spot on gel (Fig. 3).
[e] Theoretical pI of translated *R. microplus* EST (using compute pI/Mw tool on http://www.expasy.org)/observed pI estimated from position of protein spot on gel (Fig. 3).
[f] Protein in UniProt database (http://www.pir.uniprot.org) or NCBI database to which respective ESTs showed significant peptide sequence identity.
[g] Not identified = no significant match (*E* value < 0.1) with any protein in NCBI databases.

sequences of TC5777 and TC9698 share a similar degree of sequence identity (43%; *E* value 3e−46) that the individual TCs share with Q4PN29. The proteins encoded by TC5777 and TC9698, therefore, may be members of a tick-specific family of proteins of yet unknown function. Three translated ESTs (TC9513, TC12042 and TC9611) remained unidentified and may encode tick-specific proteins that have not yet been described. Three proteins (accession no. Q4PLZ7, P27463, and the protein encoded by TC9513) were detected in two protein bands each, suggesting different isoforms or posttranslational modifications. Two sets of peptides from protein bands B9 and B16 did not match any translated *R. microplus* EST. Instead they directly matched proteins in databases available through NCBI/BLAST/blastp, one to *R. microplus* actin (accession no. Q6X4V4), the other to a protein-L-isoaspartate(D-aspartate) *O*-methyltransferase (accession no. P15246), a highly conserved protein, which could be of bovine or tick origin. Several protein bands yielded peptide spectra that matched to different proteins, indicating that these bands contained more than one protein. Several protein bands also contained traces of bovine proteins or their degradation products (see below).

In a second set of experiments, pooled MicroRotofor fractions were subjected to an additional IEF step on IPG strips of the respective pH range, and proteins were then separated by mass on a second dimension gradient gel. Fig. 4 shows membrane-associated proteins extracted from *Babesia*-infected tick midguts, and is composed of 3 representative gels with 50 μg protein each on IPG strips of overlapping pH ranges (3–6, 5–8 and 7–10). Of thirty differentially expressed proteins that were submitted to MS/MS analysis, ten contained identifiable proteins that were likely to be of tick origin (Table 2). As in the first set of experiments, several protein spots yielded peptide spectra that matched different proteins, indicating that the spot contained more than one protein. One protein (accession no. P27463) was detected in two adjacent 2-D gel spots (Fig. 4, spots S8 and S9). The same protein had also been detected in two adjacent bands in the first set of MS/MS experiments with 1-D gel analysis of Micro-Rotofor fractions (Fig. 3, bands 10 and 12). This midgut protein may be present in several isoforms or contain different posttranslational modifications, at least two of which appear to be up-regulated in response to infection with *B. bovis*. Three sets of peptides from protein spots S2, S5 and S6 did not match any translated *R. microplus* EST, but directly matched proteins in databases available through NCBI/BLAST/blastp. One matched

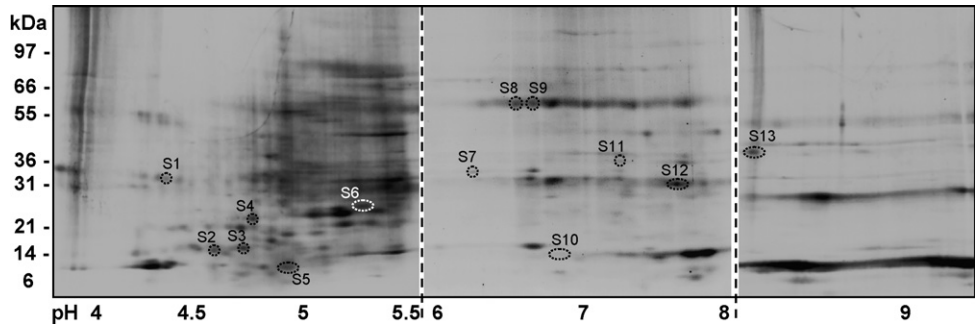

Fig. 4. Two-dimensional separation of midgut membrane-associated proteins contained in MicroRotofor fractions 1–10. A representative analysis of proteins extracted from *Babesia*-infected *R. microplus* females is shown. The circled differentially expressed proteins S1–S13 were analyzed by MS/MS, and the results presented in Table 2. Proteins were fractionated by MicroRotofor. Selected fractions were pooled and submitted to IEF on narrow pH range IPG strips (pH 3–6, 5–8, and 7–10; horizontal axis), followed by separation by mass on 4–20% gradient polyacrylamide gels (vertical axis). Molecular masses in kDa are indicated on the left.

R. microplus actin (accession no. Q6X4V4), which was also found in the first set of experiments (Table 2), confirming its infection-induced up-regulation in tick midguts. The other two proteins were identified as a highly conserved peroxiredoxin-6/antioxidant protein 2 (accession no. O08709), which may be of bovine or of tick origin, and a bacterial major MR/P fimbria protein precursor (accession no. Q03011). Several 2-D gel spots contained bovine proteins or their degradation products (see below).

3.3. Functional classification of up/down-regulated tick midgut proteins

Sixteen identified proteins from both sets of experiments were functionally classified as metabolic, signaling, molecular chaperone, cytoskeletal or mixed function proteins (Table 3). Since we were mainly interested in identifying proteins that may be of use for the development of novel tick control strategies, we omitted proteins that might be of bovine origin or proteins that are highly conserved within the animal kingdom. Proteins that were up-regulated in midguts of *B. bovis*-infected ticks turned out to be primarily of metabolic or signaling function. Four of the classified proteins in Table 3 (proteins encoded by TC5764, TC6908, TC9038 and *R. microplus* actin Q6X4V4) were identified in both sets of experiments, further confirming their up-regulation in infected ticks.

An additional four proteins listed in Table 3 could not be functionally classified because of a lack of published information. One of these proteins (encoded by TC12042) was identified in both sets of experiments, confirming its up-regulation in infected ticks. These presumably tick-specific proteins were all up-regulated in response to infection of ticks with *B. bovis* and may be of interest as potential targets for the development of control strategies. We are planning to rerun database searches for these proteins as genome data and TC annotation data for *R. microplus* become more complete.

3.4. Bovine proteins in tick midgut extracts

Since blood meal digestion in ticks is intracellular, tick midgut extracts contained a mixture of tick proteins and bovine blood proteins. Bovine proteins were abundant in both sets of analyzed protein samples and most of them were found in multiple protein bands or spots, indicating presence of intact protein and various digestive products. The most abundant bovine proteins found during the MS/MS analysis were serum albumin precursor (accession no. P02769), hemoglobin beta subunit (P02070), hemoglobin beta subunit, fetal (P02081), fibrinogen gamma-B chain precursor (P12799), fibrinogen beta chain precursor (P02676), annexin AI (P46193), flavin reductase (P52556), carbonic anhydrase 2 (P00921), and apolipoprotein A-I precursor (P15497).

4. Discussion

The aim of this study was to increase our knowledge about potential antigenic targets in the *R. microplus* midgut, with particular emphasis on membrane proteins that are differentially expressed in response to infection with *B. bovis*. We were able to identify sixteen proteins from *R. microplus* midgut membranes (Table 3) as potential candidates for further studies aimed to develop novel tick control strategies that may affect both the tick and the pathogen transmitted by it. Table 3 lists only proteins that are likely to be of tick origin. Any proteins

Table 2
R. microplus midgut proteins up/down-regulated in *Babesia*-infected females, found in pooled MicroRotofor fractions run on 2-D gels

Spot #	Up/down-regulation	Match to *R. microplus* translated EST[a]	Peptide sequences[b]	% Coverage[c]	Mr (kDa)[d] theoretical/observed	pI[e] theoretical/observed	EST match to GenBank accession no.[f]	*E* value	Protein ID [f]
S2	(+)	TC5954	DGNGYISSAELR; ALGQNPTEADVKK; ALGQNPTEADVK; HLLTTLGEK;	23	17.2/∼18	4.78/∼4.5	Q4PM87	6e−73	Nonmuscle myosin essential light chain [*Ixodes scapularis*]
S2	(+)	TC9038	FAAAAAPAAGGGAAAAKPEESKK; SAFVEGVR;	10	34.8/∼18	5.87/∼4.5	Q4PMB4	2e−144	60S acidic ribosomal protein P0 [*Ixodes scapularis*]
S2	(+)	–	LGTPSAAQNLR; NAAVVITDAGGK;	13	17.9/∼18	4.77/∼4.5	Q03011	n/a	Major MR/P fimbria protein precursor
S3	(+)	TC5888	NVLAGPFPEEQK; DKDmGTHADFLEGLK; SLDLRPTNATIEK;	25	18.0/∼19	4.62/∼4.6	AAV41826	3e−72	Myosin alkali light chain protein [*Haemaphysalis qinghaiensis*]
S4	(+)	TC9422	AGGEAAELR; ASDFANQHT; NHDGYISYPELR;	19	17.1/∼23	4.70/∼4.6	Q4PM41	1e−31	Multiple coagulation factor deficiency 2-like [*Ixodes scapularis*]
S5	(+)	–	AGFAGDDAPR; DLTDYLmK; GYSFTTTAER;	7	41.7/∼14	5.3/∼4.8	Q6X4V4	n/a	Actin [*Boophilus microplus*]
S6	(+)	–	LPFPIIDDK; LSILYPATTGR; NFDEILR; VVFIFGPDKK;	17	24.9/∼26	6.02/∼5.4	O08709	n/a	Peroxiredoxin-6 (antioxidant protein 2) (1-Cys peroxiredoxin)
S8	(+)	TC5764	ILGLIESGK; LADLLER; VSFTGSTEVGK; VNLELGGK;	8	46.2/∼55	5.79/∼6.7	P27463	2e−139	Aldehyde dehydrogenase 1A1 [*Gallus gallus*]
S9	(+)	TC5764	ILGLIESGK; LADLLER; VSFTGSTEVGK; VNLELGGK; LIQEAAGR;	10	46.2/∼55	5.79/∼6.8	P27463	2e−139	Aldehyde dehydrogenase 1A1 [*Gallus gallus*]
S11	(+)	TC12042	DILAVSIER; GFSFHIR; IDPLTTR;	10	24.6/∼35	9.01/∼7.3	–	–	n.i.[g]
S11	(+)	TC6908	FPETVLSASR; DETSYGIPK;	9	36.1/∼35	7.09/∼7.3	Q4PM32	0.0	Guanine nucleotide-binding protein [*Ixodes scapularis*]
S7	(−)	TC5830	EFTQAVEmK; mYTTLGVDYDER; AAVITAEGDSQAAALLAK; VLPSITNEVLK; NVPVVTGSK; ILFRPVQEQLPR; AFGEAGDALVELR;	27	34.7/∼34	6.92/∼6.4	Q2F5J2	2e−120	Prohibitin protein WPH [*Bombyx mori*]
S12	(−)	TC6136	FLVEGK; YENIVK; LLVQKR; SKYENIVK;	10	21.9/∼31	7.22/∼7.5	Q4PMC5	3e−79	Signal sequence receptor beta [*Ixodes scapularis*]

[a] Translated EST from a *Rhipicephalus* (*Boophilus*) *microplus* EST database (Guerrero et al., 2007) to which significant peptide sequence similarity was found.
[b] Sequence information obtained from capillary-HPLC-electrospray tandem mass spectrometry. Probabilities of peptide ID were ≥95%.
[c] % Coverage of the respective EST provided by the identified peptide sequences.
[d] Theoretical molecular mass of translated *R. microplus* EST (using compute pI/Mw tool on http://www.expasy.org)/observed mass estimated from position of protein spot on gel (Fig. 4).
[e] Theoretical pI of translated *R. microplus* EST (using compute pI/Mw tool on http://www.expasy.org)/observed pI estimated from position of protein spot on gel (Fig. 4).
[f] Protein in NCBI database to which respective ESTs showed significant peptide sequence identity.
[g] Not identified.

Table 3
Functional classification of selected up/down-regulated *R. microplus* midgut proteins

Spot #	Up/down-regulation	Match to *R. microplus* translated EST[a]	Accession number[b]	Protein family[c]	Molecular function[c]	Biological processes[c]
1. Metabolism functional class						
B2	(+)	TC7442	Q7PXY3	NADH dehydrogenase (ubiquinone) chain 11	Electron carrier activity; oxidoreductase activity, acting on NADH or NADPH; iron ion binding; NADH dehydrogenase (ubiquinone) activity; oxidoreductase activity	Mitochondrial electron transport, NADH to ubiquinone; electron transport
B7	(+)	TC6480	Q4PM57	Mitochondrial H(+)-ATP synthase, subunit B, eukaryotic type	Hydrogen ion transporting ATP synthase activity, rotational mechanism	ATP synthesis coupled proton transport
B8, S2	(+)	TC9038	Q4PMB4	Acidic ribosomal protein P0	Structural constituent of ribosome	Ribosome biogenesis and assembly; translational elongation
B10, B12, S8, S9	(+)	TC5764	P27463	NAD-dependent aldehyde dehydrogenase	Retinal dehydrogenase activity; oxidoreductase activity	Binds free retinal and cellular retinol-binding protein-bound retinal. Can convert/oxidize retinaldehyde to retinoic acid
B14	(+)	TC12227	XP_782614	Ribophorin I	Dolichyl-iphosphooligosaccharide-protein glycotransferase activity	Protein amino acid glycosylation; essential subunit Ribophorin I of oligosaccharyltransferase (OST); OST catalyses the transfer of an oligosaccharide from dolichol pyrophosphate to selected asparagine residues of nascent polypeptides as they are translocated into the lumen of the rough endoplasmic reticulum
2. Signaling functional class						
B13, S11	(+)	TC6908	Q4PM32	WD-repeat proteins	Guanine nucleotide-binding protein	Found in all eukaryotes; implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions. The specificity of the proteins is determined by the sequences outside the repeats themselves. Examples of such complexes are G proteins (beta subunit is a beta-propeller), TAFII transcription factor, and E3 ubiquitin ligase
S2	(+)	TC5954	Q4PM87	Calmodulin; EF-hand	Calcium ion binding	Ca^{2+}-binding protein; involved in signal transduction mechanisms/cytoskeleton/cell division and chromosome partitioning

S3	(+)	TC5888	AAV41826	EF-hand	Calcium ion binding	Ca^{2+}-binding protein; involved in signal transduction mechanisms/cytoskeleton/cell division and chromosome partitioning
S4	(+)	TC9422	Q4PM41	EF-hand	Calcium ion binding	Ca^{2+}-binding protein; involved in signal transduction mechanisms/cytoskeleton/cell division and chromosome partitioning
B7, B11	(+)	TC8929	Q4PLZ7	Signal peptidase complex, SPC3 subunit	Signal peptidase activity	Signal peptide processing
S12	(−)	TC6136	Q4PMC5	Translocon-associated protein, beta subunit	Receptor activity	Protein retention in ER; vesicle-mediated transport; cotranslational protein targeting to membrane
3. Molecular chaperone functional class						
B1	(−)	TC12726	Q0MUU6	Chaperone HSP70	ATP binding	Protein folding; response to unfolded protein
B15	(−)	TC6772	Q86G69	Alpha-crystallin-related small heat shock protein	Protein binding	Protein folding; response to unfolded protein
4. Cytoskeleton functional class						
B9, S5	(+)	–	Q6X4V4	Actin	ATP binding; protein binding; nucleotide binding; structural molecule activity	Highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells
B5	(−)	TC7873	XP_396670	Lamin	Structural molecule activity	Components of the cytoskeleton and the nuclear envelope
5. Miscellaneous functional class						
S7	(−)	TC5830	Q2F5J2	Membrane protease subunits, stomatin/prohibitin homologs	Protein binding; serine-type endopeptidase activity; receptor activity; receptor binding; structural constituent of cytoskeleton; transcriptional activator activity; transcriptional repressor activity	Highly conserved multifunctional protein. Vital for normal development in insects. Chaperones involved in stabilization of mitochondrial proteins. May function as surface-binding sites in plasma membrane. May be involved in regulation of cell proliferation and apoptosis. In mammals implicated in various disease states (Mishra et al., 2005)
6. Function unknown						
B13, S11	(+)	TC12042	n.i.	n/a	n/a	n/a
B16	(+)	TC5777, TC9698	Q4PN29	n/a	n/a	n/a
B16	(+)	TC9611	n.i.	n/a	n/a	n/a
B3, B6	(+)	TC9513	n.i.	n/a	n/a	n/a

[a] Translated EST from a *Rhipicephalus* (*Boophilus*) *microplus* EST database (Guerrero et al., 2007) to which significant peptide sequence similarity was found.

[b] UniProt or GenBank accession number.

[c] According to information obtained from the Gene Ontology Database (http://www.geneontology.org/), databases at UniProt (http://www.pir.uniprot.org/), Pfam (http://www.sanger.ac.uk/Software/Pfam) or InterPro (http://www.ebi.ac.uk/interpro) and literature search.

that could be of bovine origin were omitted from further characterization and from consideration as potential targets for tick control.

Four of the up-regulated 2-D gel spots reported in Table 3 could not be functionally characterized and will need to be reexamined once the EST databases for *R. microplus* become more complete. Three of these proteins matched translated *R. microplus* ESTs (TC9513, TC9611 and TC12042), but did not match to any protein in online available databases. The proteins encoded by TC5777 and TC9698 matched exclusively to an *Ixodes* putative salivary secreted protein (accession no. Q4PN29), and may be members of a tick-specific family of proteins of yet unknown function. When compared to each other, the translated sequences of TC5777 and TC9698 share a similar degree of sequence identity (43%; *E* value 3e−46) that the individual TCs share with Q4PN29. We had previously observed that in *R. microplus* ovaries, *Babesia* infection induced an increase in expression of three proteins (encoded by TC9227, TC11172 and TC12656) that appear to be members of the same protein family as Q4PN29 and the proteins encoded by TC5777 and TC9698 (Rachinsky et al., 2007). All members of this protein family appear to contain a large number of arginine, glycine and proline residues, a R+GDC domain and a pair of cysteine residues in apparently conserved locations within the molecule. These characteristics and their small size suggest that they might belong to a group of invertebrate antimicrobial peptides (Bulet et al., 2004) and that they may be involved in host defense mechanisms, since expression of Q4PN29-related proteins was also elevated in the ovaries of *Babesia*-infected ticks (Rachinsky et al., 2007).

Among the proteins that were expressed at higher levels in response to infection of *R. microplus* with *B. bovis* were five metabolic enzymes (Table 3). Expression changes in the proteins encoded by TC7442 and TC6480 may induce changes in electron and proton transport, which are likely to affect midgut physiology, including endocytosis/phagocytosis involved in uptake of blood meal components, as well as diuresis and water balance. In ixodid ticks, the blood meal is concentrated by removing excess water and electrolytes in an energy-dependent process, resulting in secretion of most of the water content of the blood meal back into the host via the salivary glands (Sonenshine, 1991; Kaufman, 2007).

Up-regulation in infected ticks of the protein encoded by TC9038, which is presumably a ribosomal protein P0, may indicate a response to cell stress. Ribosomal protein P0 is a conserved protein which provides a structural component of the ribosomal stalk of the large subunit. In *Drosophila*, ribosomal protein P0 also has endonuclease activity, suggesting possible roles in DNA repair (Yacoub et al., 1996). Various sources of stress, including starvation and cell density, changed expression of ribosomal protein P0 in *Aedes albopictus* mosquito cells. Furthermore, expression of *A. albopictus* P0 RNAi was associated with increased apoptosis (Jayachandran and Fallon, 2003). In *R. microplus,* two translated ESTs, TC9038 and TC9039, encode two structurally related (74% sequence identity; *E* value 2e−116) ribosomal P0 proteins. The protein encoded by TC9038 matches to an *I. scapularis* protein (accession no. Q4PM57; Table 3), whereas the protein encoded by TC9039 matches to the *A. albopictus* P0 protein mentioned above (accession no. Q8MQT0). Serial analysis of gene expression in *R. microplus* larvae from organophosphate susceptible and resistant tick strains revealed reduced levels of TC9039/ribosomal protein P0 gene expression in resistant ticks, while other ribosomal proteins were expressed at higher levels (Guerrero et al., 2007). This indicates that in ticks, several of the ribosomal proteins may have other functions, in addition to providing structural components of the ribosomes. A recent study by Terkawi et al. (2007) described a *Babesia gibsoni* ribosomal protein P0 that induced cross-protective immunity against *B. microti* in mice.

The best match for the protein encoded by TC5764 was chicken aldehyde dehydrogenase 1A1 (accession no. P27463), characterized as having oxidoreductase and retinal dehydrogenase activities. This enzyme belongs to a large family of conserved oxidoreductases. These enzymes can interact with a large variety of potential substrates, including retinal and retinaldehyde, which can be oxidized to retinoic acid. The multiple roles that retinoic acid plays in vertebrate development are well documented (Gilbert, 2006), but considerably less is known about its roles and distribution in invertebrates. The protein encoded by TC5764 also shows a high degree of sequence identity with a mosquito aldehyde dehydrogenase with oxidoreductase activity (accession no. Q7Q165, *E* value 7e−138).

One of the up-regulated metabolic proteins, encoded by TC12227, matched ribophorin I (Pfam accession no. PF04597), which is an endoplasmic reticulum-associated essential subunit of oligosaccharyltransferase (OST). The hetero-oligomeric protein complex OST is involved in protein glycosylation, catalyzing the transfer of oligosaccharides to specific asparagine residues of nascent polypeptides as they are translo-

cated into the lumen of the rough endoplasmic reticulum (Fu and Kreibich, 2000; Hardt et al., 2000). The best match for the protein encoded by TC12227 was an echinodermal ribophorin I (accession no. XP_782614; *E* value 2e−56). Matches with alignment scores in a similar range were found to a variety of eukaryotic ribophorin I subunits, including *Bos taurus* hypothetical protein LOC507939 (accession no. A3KN04; *E* value 4e−53) and *Aedes aegypti* ribophorin (accession no. Q16K94; *E* value 8e−22), indicating the conserved nature of the protein. Mammalian ribophorin I appears to act as a chaperone to dramatically enhance the *N*-glycosylation of selected membrane proteins by the catalytic subunit STT3 of OST (Wilson and High, 2007). The increase in ribophorin I-like protein indicates increased levels of protein glycosylation in the midgut of *Babesia*-infected ticks, but we do not know at this point if an increase in *N*-glycosylation ultimately poses an advantage for the *Babesia* parasites. For many proteins *N*-glycosylation is a requirement for correct folding and function. The only currently registered recombinant vaccines against *R. microplus* are based on the midgut glycoproteins Bm86 and Bm95 (Willadsen, 2004; de la Fuente et al., 2006a). So far, all characterized digestive proteins of hard ticks appear to be *N*-glycosylated (Mulenga et al., 2000, 2003; Boldbaatar et al., 2006; Motobu et al., 2007; Sojka et al., 2007; Alim et al., 2007), and most identified anti-tick vaccine antigen candidates are glycoproteins as well (Mulenga et al., 2000; de la Fuente et al., 2006a). Glycosylated recombinant proteins are more immunogenic than non-glycosylated ones, providing additional evidence that sugar epitopes on tick proteins may enhance the protective capacity of tick vaccine antigen preparations (de la Fuente et al., 2006a). The importance of glycosylated midgut proteins for blood meal digestion, development and reproduction in the tick is well documented, but information about the importance of these proteins for pathogen transmission is just starting to emerge. In *Anopheles stephensi*, the monoclonal antibody MG96 blocks *Plasmodium yoelii* development by binding to sugar epitopes of *A. stephensi* midgut protein, indicating that glycosylated midgut proteins may provide alternative targets for the development of transmission-blocking vaccines (Dinglasan et al., 2003). Using the anti-carbohydrate malaria transmission-blocking monoclonal antibody MG96, a conserved carbohydrate target has recently been identified in the midguts of several arthropod vectors, including the tick *Dermacentor variabilis*, further emphasizing the role of protein–carbohydrate recognition mechanisms in vector–host pathogen interactions (Dinglasan et al., 2005; Dinglasan and Jacobs-Lorena, 2005). In the light of these findings, one could speculate that targeting the tick's glycosylation machinery, including enzymes like the differentially expressed ribophorin I mentioned above, may affect the tick vector as well as the pathogens transmitted by it. It is also interesting to note that N-linked glycans of invertebrates have many characteristics in common, but often differ greatly from N-linked glycans in vertebrates (Wilson, 2002). N-linked glycans of invertebrates are therefore considered promising targets for the development of pan-arthropod vaccines (Mejia et al., 2006). As will be discussed below in more detail, ribophorin I may also be an integral part of ribosome receptors that are associated with sites of cotranslational protein translocation across the membrane of the endoplasmic reticulum (Johnson and van Waes, 1999). The importance of specific surface glycoproteins for parasite invasion has recently also gained more attention. In vitro studies using different *Babesia* species and vertebrate red blood cells indicated an important role of sialic acid and/or sialoglycoproteins for invasion of erythrocytes (Gaffar et al., 2003; Takabatake et al., 2007). Of particular interest is the finding that glycophorin A-knockout mice, which lack sialoglycoproteins on the red blood cell membrane, were resistant to lethal infection of *Babesia rodhaini* (Takabatake et al., 2007).

Six proteins that were differentially expressed in response to infection of *R. microplus* with *B. bovis* appear to be involved in signaling processes (Table 3). Four of these proteins may be involved in receptor-mediated signal transduction pathways in midgut cells. The protein encoded by TC6908 matched closely to a guanine nucleotide-binding protein of *I. scapularis* (Q4PM32) and, due to the conserved sequence of this protein, also matched to a large number of guanine nucleotide-binding proteins from a variety of organisms. The proteins encoded by TC5954, TC5888 and TC9422 matched proteins that contain EF-hand Ca^{2+}-binding motifs. Some EF-hand proteins act as Ca^{2+} buffers, modulating intracellular Ca^{2+} concentration. The majority of EF-hand proteins act as Ca^{2+} sensors, which translate the signal represented by an increased intracellular Ca^{2+} concentration into diverse biochemical responses within a cell (Gifford et al., 2007). The up-regulation of these four proteins may therefore indicate an infection-induced increase in Ca^{2+} and G protein-related signaling activity. Apicomplexan parasites, a group of protozoan parasites which includes *Babesia*, *Plasmodium*, *Toxoplasma* and *Cryptosporidium*, actively penetrate host cells using an efficient system of adhesion-based and actin-dependent motility.

Invasion of mammalian host cells is facilitated by secretion of adhesins from apically located secretory organelles which likely is triggered by a coinciding sharp rise in intracellular Ca^{2+} concentration in the apicomplexan parasite (reviewed in Sibley, 2004; Soldati et al., 2004). Changes occurring within the apicomplexan parasites during invasion of mammalian host cells and invasion-induced changes in mammalian cell physiology have been fairly well characterized (reviewed in Cooke et al., 2004; Soldati et al., 2004; Nagamune et al., 2007). Little has been reported about invasion of apicomplexan parasites into invertebrate midgut cells or invasion-induced changes in midgut cell physiology. According to a detailed electron microscopic study, *Babesia microti* invades *Ixodes* midgut cells through a combination of active penetration and invagination by the host cell (Rudzinska et al., 1983). Our results indicate that *B. bovis* invasion of *R. microplus* midgut cells stimulates the synthesis of proteins that are involved in Ca^{2+} and G protein-related signaling, suggesting these changes are important for successful pathogen transmission to the invertebrate host. It is interesting to note that in acaricide-resistant and acaricide-susceptible strains of *R. microplus*, gene expression of the above-mentioned signaling component represented by TC5954 appears to be consistently up-regulated in response to acaricide treatment (Guerrero et al., 2007, unpublished data). This may indicate that expression of TC5954 is up-regulated in response to a variety of stress conditions.

Two of the signaling proteins that were differentially expressed in response to infection of *R. microplus* with *B. bovis* appear to be involved in processes related to protein folding and protein translocation from the cytoplasm into the endoplasmic reticulum (ER) lumen. The protein encoded by TC8929 appeared to be up-regulated in infected ticks. It matched closely to a 22 kDa subunit of an endoplasmic reticulum signal peptidase from *I. scapularis* (accession no. Q4PLZ7). ER signal peptidases are associated with the protein translocation machinery, the translocon, of the endoplasmic reticulum (Johnson and van Waes, 1999). These peptidases cleave the N-terminal signal sequences of secretory or membrane proteins while these proteins are translocated into the lumen of the ER, thereby controlling protein transport and localization within the cell (Paetzel. et al., 2002). The *Ixodes* protein showed similarity to the eucaryotic signal peptidase complex (SPC) subunits SPC3 from yeast and its mammalian homolog SPC22/23. At this point, we do not know if an increase in expression of this particular signal peptidase subunit results in higher signal peptidase activity and how that may affect the tick host. In yeast, SPC3 may be directly involved in the cleavage of signal peptides and is essential for cell viability, but its overexpression did not result in higher signal peptidase activity (Meyer and Hartmann, 1997). Peptidases are popular and promising drug targets, especially peptidases which are unique to parasites or pathogens of interest with low similarity to those of vertebrates (Rawlings, 2007). The sequence of the presumed signal peptidase subunit encoded by TC8929 matched considerably better to the *Ixodes* signal peptidase (E value 1e−88) than to respective insect (E values $\geq$ 2e−71) or vertebrate proteins (E values $\geq$ 2e−60). The protein encoded by TC6136 appeared to be down-regulated in infected tick midgut. It matched an *I. scapularis* protein (accession no. Q4PMC5) which has been characterized as the beta subunit of a translocon-associated protein. Proteins belonging to this family (Pfam accession no. PF05753) are thought to aid the translocation of nascent polypeptides into the lumen of the ER. The *I. scapularis* protein belongs to a cluster of ten proteins that share at least 50% sequence identity (http://www.pir.uniprot.org; cluster CG5474-PA). All members of this cluster are arthropod proteins, indicating that the *I. scapularis* protein and the protein encoded by TC6136 are more closely related to arthropod proteins than to the respective vertebrate members of the family of translocon-associated proteins. Due to their closer similarity to arthropod members than to vertebrate members of their respective protein families, the translocon-associated proteins encoded by TC8929 and TC6136 may have potential as protein targets that could be exploited for the development of tick control strategies. One potential problem with using a protein antigen from a family of highly conserved proteins is the possibility of inducing autoimmune responses within the mammalian host. Because of their similarity to arthropod proteins, tick translocon-associated proteins should pose a lower risk for inducing autoimmune responses when used for immunizing a mamalian host. It is interesting to note that the earlier discussed *N*-glycosylation promoting protein ribophorin I may be an integral part of ribosome receptors that are associated with the translocons in ER membranes (Johnson and van Waes, 1999). The previously mentioned differentially expressed ribosomal protein P0 may be involved in translational elongation and, thus, it appears that at least four tick proteins that are involved in protein elongation, modification and translocation into the ER lumen (TC12227, TC9038, TC8929, TC6136) might be differentially expressed in response to infection with

Babesia parasites. Since targeting components of the complex and highly coordinated translocation machinery in the tick ER may have negative effects on the tick and also may interfere with pathogen transmission in the midgut, these proteins are potential targets for the development of tick control methods. Since *R. microplus* females take up bovine antibodies with their blood meal and blood meal digestion occurs within the midgut cells, bovine immunoglobulins should be able to target intracellular proteins within these midgut cells. Intracellular tick midgut proteins may, therefore, be suitable candidates for vaccine development, even if they are conserved proteins. As shown for subolesin, conserved intracellular proteins may be used in anti-tick vaccines (de la Fuente et al., 2006b). It has also been shown that – as discussed below in more detail – even the highly conserved heat shock proteins have recently gained more attention as candidates for vaccine development (Silva, 1999; Acharya et al., 2007).

The apparent down-regulation of two heat shock proteins (HSP), encoded by TC12726 and TC6772, provides further indication that infection of *R. microplus* with *Babesia* might affect processes related to protein modification and processing in the tick's midgut tissue. The best match for the protein encoded by TC12726 was an insect HSP70 (Q0MUU6). The major physiological functions of members of the HSP70 superfamily are protein folding, unfolding and translocation, and assembly and disassembly of oligomeric protein complexes (Kaufman, 1990b). The protein encoded by TC6772 matched best to a *D. variabilis* HSP (Q86G69) of the α-crystallin-related small HSP family and is, therefore, also likely to function as a molecular chaperone in protein folding reactions and in particular may protect proteins from aggregation (Horwitz, 1992; Jakob et al., 1993). HSPs are produced as a protective response of cells to a variety of stress factors. In host–parasite relationships, HSPs are used by the host and by the parasite to protect themselves from adverse effects of their interaction (Kaufmann, 1990a). HSPs also provide attractive targets for immune responses towards pathogens (Maresca and Kobayashi, 1994). Despite the fact that they are highly conserved proteins, there appears to be sufficient sequence divergence between HSPs from different organisms to allow epitope recognition and host immune responses and, thus, HSPs have recently gained more attention regarding their potential as vaccine candidates as well as drug targets (Acharya et al., 2007). HSP synthesis is usually stimulated by parasite invasion (Kaufmann, 1990b; Maresca and Kobayashi, 1994) and it was, therefore, unexpected to see a decrease in two HSPs in *Babesia*-infected tick midguts. Further experiments will be needed to confirm that these particular HSPs are indeed down-regulated in response to an infection of the ticks with *Babesia*. Down-regulation of host HSPs may indicate that the *Babesia* parasites may be able to suppress a protective response in the tick host or may interfere with the host's immune response.

Among the proteins that appeared to be differentially expressed in response to *Babesia* infection were two cytoskeleton components. *R. microplus* actin (Q6X4V4) was up-regulated, whereas the protein encoded by TC7873 and matching an insect lamin (XP_396670) was down-regulated. Invasion of host cells by apicomplexan protozoan parasites usually involves changes in the parasite's cytoskeleton, but its effects on the host's cytoskeleton are diverse. *Toxoplasma* invasion of mammalian cells is dependent on actin filaments in the parasite but not on those of the host cell, whereas *Theileria* and *Cryptosporidium* invasion requires the host's actin cytoskeleton for invasion (reviewed in Mota and Rodriguez, 2002). Considerably less is known about the effects that protozoan invasion have on the actin cytoskeleton of invertebrate host cells. *Plasmodium* invasion of epithelial cells in the mosquito midgut induces remodeling of the actin cytoskeleton by proteolytic actin cleavage or by activating actin depolymerization, resulting in characteristic morphological changes (Han and Barrillas-Mury, 2002). Other changes that were observed in mosquito midgut cells in response to *Plasmodium* invasion were induction of nitric oxide synthase expression and activation of a caspase-3-like protease, indicating that host cell death which ultimately occurs is apoptotic (Zieler and Dvorak, 2000; Han and Barrillas-Mury, 2002). Mosquito epithelial responses are not universal: different actin-mediated repair mechanisms were found in different mosquito–*Plasmodium* combinations (Gupta et al., 2005). Our results suggest that actins play a role in the response of tick midgut cells to *Babesia* invasion. Unfortunately, actins are highly conserved and ubiquitously expressed in eukaryotic cells and thus are unlikely to be good target antigens. Alignment of the amino acid sequences for *R. microplus* actin and bovine actin revealed 98% sequence identity (*E* value 0.0), and similar high levels of sequence identity exist between *R. microplus* actin and actins of other vertebrates.

The down-regulated cytoskeleton protein encoded by TC7873 matched more closely to insect lamins (*E* values 2e−85 to 1e−68) than to vertebrate lamins (*E* values $\geq$ 3e−64). Lamins are the scaffold proteins of the nuclear envelope. The observed decrease in lamin

expression in *Babesia*-infected ticks may be the result of parasite-induced cleavage of nuclear lamins and a sign of apoptotic cell death of the midgut cells. In mosquito midgut, extensive damage is inflicted by invading *Plasmodium* parasites that leads to genome fragmentation and cell death (Han and Barrillas-Mury, 2002). Electron microscope studies showed that *Plasmodium* ookinete invasion was associated with morphological changes in mosquito midgut cells, among them condensation and fragmentation of the nucleus (Baton and Ranford-Cartwright, 2004). A variety of pathogenic microorganisms cause apoptotic cell death of their eukaryotic host cells, which involves cleavage of lamins leading to nuclear shrinkage and fragmentation (Fink and Cookson, 2005). The role of lamin proteolysis in eukaryotic apoptosis has been well documented, and involves specific caspases that cleave lamin to signature apoptotic fragments (Lazebnik et al., 1995; Orth et al., 1996), thereby facilitating nuclear breakdown (Rao et al., 1996).

Further indication for an involvement of apoptotic cell death in the tick midgut response comes from the observation that the level of a prohibitin-like protein, encoded by TC5830, is decreased in *Babesia*-infected ticks. The best match for TC5830 is a *Bombyx mori* prohibitin (accession no. Q2F5J2; *E* value 2e−120). Due to the conserved nature of prohibitins, high levels of sequence identity also existed with mammalian prohibitins, among them a bovine prohibitin (accession no. Q3T165; *E* value 4e−114), and with prohibitins from protozoan parasites, including *Plasmodium falciparum* (accession no. NP_700618; *E* value 1e−64) and *Theileria parva* (accession no. Q4N9C6; *E* value 3e−67). Prohibitins are multifunctional proteins that are present in multiple cellular compartments and have been implicated in regulation of cell proliferation and apoptosis, controlling such dissimilar processes as development, senescence and tumor suppression (McClung et al., 1995; Mishra et al., 2006). In insects, prohibitins are vital for normal development. In addition to acting as chaperone proteins in the mitochondria and maintaining mitochondrial function, prohibitins also appear to modulate transcriptional activity in the nucleus by interacting with various transcription factors (Mishra et al., 2006). The diverse functions of prohibitins and emerging evidence that in mammalian systems, their function can be modulated specifically in certain tissues, suggests that targeting prohibitins might be a useful therapeutic approach for the treatment of various disease states (Mishra et al., 2005). Prohibitin has recently been shown to be down-regulated in human and animal models of inflammatory bowel disease, resulting in lowered protection against oxidative stress in intestinal epithelial cells (Theiss et al., 2007). It is tempting to speculate that the decrease in prohibitin that we see in *Babesia*-infected ticks may reflect similar pathological conditions caused by parasite invasion into midgut epithelial cells as the ones described for disease-impaired mammalian digestive tract cells.

5. Conclusions

Our data support the existence of distinct infection-induced changes in protein expression in the midgut epithelium of *R. microplus*. Similar midgut epithelial responses were observed in *Anopheles* midgut during *Plasmodium* invasion (Vlachou et al., 2005). In both host–parasite systems, major responses included changes in the expression of actin and cytoskeleton components, apoptosis factors, and components of redox metabolism and intracellular signaling pathways. Altered patterns of gene expression in the tick midgut in response to infection with *B. bovis*, especially in such fundamental processes as these, may be essential for establishment and survival of the parasite and therefore may provide ideal targets for vaccines intended to block transmission. Further experiments aimed at elucidating the functions of selected up- and down-regulated proteins will provide us with insights regarding the interactions that occur during pathogen transmission between the *Babesia* parasite and the tick host, and may provide us with suitable target protein candidates for the development of novel tick control methods.

We are just beginning to explore molecular aspects of the interactions between ticks and the pathogens transmitted by them (Ramamoorthi et al., 2005; Sukumaran et al., 2006; Foley and Nieto, 2006). Understanding tick–parasite interactions will facilitate the development of control methods that affect tick vector and pathogen transmission. In that sense, the differentially expressed proteins identified in this study will be used for further investigations aimed to determine their functions, in particular the possibility of their involvement in pathogen transmission in *R. microplus*.

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References

Acharya, P., Kumar, R., Tatu, U., 2007. Chaperoning a cellular upheaval in malaria: heat shock proteins in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 153, 85–94.

Alim, M.A., Tsuji, N., Miyoshi, T., Islam, M.K., Huang, X., Motobu, M., Fujisaki, K., 2007. Characterization of asparaginyl endopeptidase, legumain induced by blood feeding in the ixodid tick *Haemaphysalis longicornis*. Insect Biochem. Mol. Biol. 37, 911–922.

Baton, L.A., Ranford-Cartwright, L.C., 2004. *Plasmodium falciparum* ookinete invasion of the midgut epithelium of *Anopheles stephensi* is consistent with the Time Bomb model. Parasitology 129, 663–676.

Bock, R., Jackson, L., De Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129, S247–S269.

Boldbaatar, D., Sikasunge, C.S., Battsetseg, B., Xuan, X., Fujisaki, K., 2006. Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis*. Insect Biochem. Mol. Biol. 36, 25–36.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bulet, P., Stöcklin, R., Menin, L., 2004. Anti-microbial peptides: from invertebrates to vertebrates. Immunol. Rev. 198, 169–184.

Cooke, B.M., Lingelbach, K., Bannister, L.H., Tilley, L., 2004. Protein trafficking in *Plasmodium falciparum*-infected red blood cells. Trends Parasitol. 20, 581–589.

da Silva Vaz Jr., I., Martinez, R.H.M., Oliveira, A., Heck, A., Logullo, C., Gonzalez, J.C., Dewes, H., Masuda, A., 1996. Functional bovine immunoglobulins in *Boophilus microplus*hemolymph. Vet. Parasitol. 62, 155–160.

de la Fuente, J., Kocan, K.M., 2006. Strategies for development of vaccines for control of ixodid tick species. Parasite Immunol. 28, 275–283.

de la Fuente, J., Canales, M., Kocan, K.M., 2006a. The importance of glycosylation in the development of novel tick vaccine strategies. Parasite Immunol. 28, 687–688.

de la Fuente, J., Almazán, C., Blas-Machado, U., Naranjo, V., Mangold, A.J., Blouin, E.F., Gortazar, C., Kocan, K.M., 2006b. The tick protective antigen, 4D8, is a conserved protein involved in modulation of tick blood digestion and reproduction. Vaccine 24, 4082–4095.

Dinglasan, R.R., Jacobs-Lorena, M., 2005. Insight into a conserved lifestyle: protein–carbohydrate adhesion strategies of vector-borne pathogens. Infect. Immun. 73, 7797–7807.

Dinglasan, R.R., Fields, I., Shahabuddin, M., Azad, A.F., Sacci Jr., J.B., 2003. Monoclonal antibody MG96 completely blocks *Plasmodium yoelii* development in *Anopheles stephensi*. Infect. Immun. 71, 6995–7001.

Dinglasan, R.R., Valenzuela, J.G., Azad, A.F., 2005. Sugar epitopes as potential universal disease transmission blocking targets. Insect Biochem. Mol. Biol. 35, 1–10.

Fink, S.L., Cookson, B.T., 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect. Immun. 73, 1907–1916.

Foley, J., Nieto, N., 2006. *Anaplasma phagocytophilum* subverts tick salivary gland proteins. Trends Parasitol. 23, 3–5.

Fu, J., Kreibich, G., 2000. Retention of subunits of the oligosaccharyltransferase complex in the endoplasmic reticulum. J. Biol. Chem. 275, 3984–3990.

Gaffar, F.R., Franssen, F.F.J., de Vries, E., 2003. *Babesia bovis* merozoites invade human, ovine, equine, porcine and caprine erythrocytes by a sialic acid-dependent mechanism followed by developmental arrest after a single round of cell fission. Int. J. Parasitol. 33, 1595–1603.

Garcia-Garcia, J.C., Montero, C., Redondo, M., Vargas, M., Canales, M., Boue, O., Rodrígez, M., Joglar, M., Machado, H., González, I.L., Valdés, M., Méndez, L., de la Fuente, J., 2000. Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. Vaccine 18, 2275–2287.

Gifford, J.L., Walsh, M.P., Vogel, H.J., 2007. Structures and metal-ion-binding properties of the Ca^{2+}-binding helix-loop-helix EF-hand motifs. Biochem. J. 405, 199–221.

Gilbert, S.F., 2006. Developmental Biology, eighth ed. Sinauer Associates Inc., Sunderland, MA.

Graham, O.H., Hourrigan, J.L., 1977. Eradication programs for the arthropod parasites of livestock. J. Med. Entomol. 13, 629–658.

Guerrero, F.D., Bendele, K.G., Chen, A.C., Li, A.Y., Miller, R.J., Pleasance, E., Varhol, R., Rousseau, M.-E., Nene, V.N., 2007. Serial analysis of gene expression in the southern cattle tick following acaricide treatment of larvae from organophosphate resistant and susceptible strains. Insect Mol. Biol. 16, 49–60.

Gupta, L., Kumar, S., Han, Y.S., Pimenta, P.F.P., Barillas-Mury, C., 2005. Midgut epithelial responses of different mosquito–*Plasmodium* combinations: the actin cone zipper repair mechanism in *Aedes aegypti*. Proc. Natl. Acad. Sci. U.S.A. 102, 4010–4015.

Han, Y.S., Barrillas-Mury, C., 2002. Implications of the Time Bomb model of ookinete invasion of midgut cells. Insect Biochem. Mol. Biol. 32, 1311–1316.

Harbers, A., Rodriguez, G., Berkelman, T., 2006. Fractionation by liquid-phase isoelectric focusing in the MicroRotofor cell: improved detection of low-abundance proteins. BioRadiations 118, 24–26.

Hardt, B., Aparicio, R., Bause, E., 2000. The oligosaccharyltransferase complex from pig liver: cDNA cloning, expression, and functional characterisation. Glycoconj. J. 17, 767–779.

Horwitz, J., 1992. α-Crystallin can function as a molecular chaperone. Proc. Natl. Acad. Sci. U.S.A. 89, 10449–10453.

Howell, J.M., Ueti, M.W., Palmer, G.A., Scoles, G.A., Knowles, D.P., 2007. Transovarial transmission efficiency of *Babesia bovis* tick stages acquired by *Rhipicephalus* (*Boophilus*) *microplus* during acute infection. J. Clin. Microbiol. 45, 426–431.

Jakob, U., Gaestel, M., Engel, K., Buchner, J., 1993. Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268, 1517–1520.

Jayachandran, G., Fallon, A.M., 2003. Ribosomal protein P0 from *Aedes albopictus* mosquito cells: cDNA cloning and analysis of gene expression. Genetica 119, 1–10.

Johnson, A.E., van Waes, M.A., 1999. The translocon: a dynamic gateway at the ER membrane. Annu. Rev. Cell Dev. Biol. 15, 799–842.

Kaufmann, S.H.E., 1990a. Heat-shock proteins: a missing link in the host-parasite relationship? Med. Microbiol. Immunol. 179, 61–66.

Kaufmann, S.H.E., 1990b. Heat shock proteins and the immune response. Immunol. Today 11, 129–136.

Kaufman, W.R., 2007. Gluttony and sex in female ixodid ticks: how do they compare to other blood-sucking arthropods? J. Insect Physiol. 53, 264–273.

Kavitha, K.T., Latha, B.R., Raj, G.D., John, L., 2007. Immunoprophylaxis of rabbits against *Rhipicephalus haemophysaloides* ticks using immunoaffinity purified 35 kDa midgut antigen. Vet. Arch. 77, 147–157.

Lazebnik, Y.A., Takahashi, A., Moir, R.D., Goldman, R.D., Poirier, G.G., Kaufmann, S.H., Earnshaw, W.C., 1995. Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. Proc. Natl. Acad. Sci. U.S.A. 92, 9042–9046.

Maresca, B., Kobayashi, G.S., 1994. Hsp70 in parasites: as an inducible protective protein and as an antigen. Experientia 50, 1067–1074.

McClung, J.K., Jupe, E.R., Liu, X.-T., Dell'Orco, R.T., 1995. Prohibitin: potential role in senescence, development, and tumor suppression. Exp. Gerontol. 30, 99–124.

Mejia, J.S., Bishop, J.V., Titus, R.G., 2006. Is it possible to develop pan-arthropod vaccines? Trends Parasitol. 22, 367–370.

Meyer, H.-A., Hartmann, E., 1997. The yeast SPC22/23 homolog Spc3p is essential for signal peptidase activity. J. Biol. Chem. 272, 13159–13164.

Miller, R.J., Davey, R.B., George, J.E., 2005. First report of organophosphate-resistant *Boophilus microplus* (Acari: Ixodidae) within the United States. J. Med. Entomol. 42, 912–917.

Mishra, S., Murphy, L.C., Murphy, L.J., 2006. The prohibitins: emerging roles in diverse functions. J. Cell. Mol. Med. 10, 353–363.

Mishra, S., Murphy, L.C., Nyomba, B.L.G., Murphy, L.J., 2005. Prohibit a potential target for new therapeutics. Trends Mol. Med. 11, 192–197.

Mota, M.M., Rodriguez, A., 2002. Invasion of mammalian host cells by *Plasmodium* sporozoites. BioEssays 24, 149–156.

Motobu, M., Tsuji, N., Miyoshi, T., Huang, X., Islam, M.K., Alim, M.A., Fujisaki, K., 2007. Molecular characterization of a blood-induced serine carboxypeptidase from the ixodid tick *Haemaphysalis longicornis*. FEBS J. 274, 3299–3312.

Mulenga, A., Misao, O., Sugimoto, C., 2003. Three serine proteases from midguts of the hard tick *Rhipicephalus appendiculatus*; cDNA cloning and preliminary characterization. Exp. Appl. Acarol. 29, 151–164.

Mulenga, A., Sugimoto, C., Onuma, M., 2000. Issues in tick vaccine development: identification and characterization of potential candidate vaccine antigens. Microbes Infect. 2, 1353–1361.

Nagamune, K., Moreno, S.N., Chini, E.N., Sibley, L.D., 2007. Calcium regulation and signaling in apicomplexan parasites. In: Burleigh, B., Soldati, D. (Eds.), Molecular Mechanisms of Parasite Invasion. Landes Bioscience, Austin, TX, pp. 1–12.

Nuttall, P.A., Trimnell, A.R., Kazimirova, M., Labuda, M., 2006. Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. Parasite Immunol. 28, 155–163.

Orth, K., Chinnaiyan, A.M., Garg, M., Froehlich, C.J., Dixit, V.M., 1996. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. J. Biol. Chem. 271, 16443–16446.

Paetzel, M., Karla, A., Strynadka, N.C.J., Dalbey, R.E., 2002. Signal peptidases. Chem. Rev. 102, 4549–4579.

Rachinsky, A., Guerrero, F.D., Scoles, G.A., 2007. Differential protein expression in ovaries of uninfected and *Babesia*-infected southern cattle ticks *Rhipicephalus* (*Boophilus*) *microplus*. Insect Biochem. Mol. Biol. 37, 1291–1308.

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X.F., Fish, D., Anguita, J., Norgard, M.V., Kantor, F.S., Anderson, J.F., Koski, R.A., Fikrig, E., 2005. The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature 436, 573–577.

Rao, L., Perez, D., White, E., 1996. Lamin proteolysis facilitates nuclear events during apoptosis. J. Cell Biol. 135, 1441–1455.

Rawlings, N.D., 2007. Unusual phyletic distribution of peptidases as a tool for identifying potential drug targets. Biochem. J. 401, e5–e7.

Sibley, L.D., 2004. Intracellular parasite invasion strategies. Science 304, 248–253.

Riek, R.F., 1966. The life cycle of *Babesia argentina* (Lignieres, 1903) (Sporozoa: Piroplasmidae) in the tick vector *Boophilus microplus* (Canestrini). Aust., J. Agric. Res. 17, 247–254.

Rudzinska, M.A., Lewengrub, S., Spielman, A., Piesman, J., 1983. Invasion of *Babesia microti* into epithelial cells of the tick gut. J. Protozool. 30, 338–346.

Silva, C.L., 1999. The potential use of heat-shock proteins to vaccinate against mycobacterial infections. Microbes Infect. 1, 429–435.

Sojka, D., Hajdušek, O., Dvořák, J., Sajid, M., Franta, Z., Schneider, E.L., Craik, C.S., Vancová, M., Burešová, V., Bogyo, M., Sexton, K.B., McKerrow, J.H., Caffrey, C.R., Kopáček, P., 2007. IrAE—An asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. Int. J. Parasitol. 37, 713–724.

Soldati, D., Foth, B.J., Cowman, A.F., 2004. Molecular and functional aspects of parasite invasion. Trends Parasitol. 20, 567–574.

Sonenshine, D.E., 1991. Biology of Ticks, vol. 1. Oxford University Press, New York.

Sukumaran, B., Narasimhan, S., Anderson, J.F., DePonte, K., Marcantonio, N., Krishnan, M.N., Fish, D., Telford, S.R., Kantor, F.S., Fikrig, E., 2006. An *Ixodes scapularis* protein required for survival of *Anaplasma phagocytophilum* in tick salivary glands. J. Exp. Med. 203, 1507–1517.

Takabatake, N., Okamura, M., Yokoyama, N., Ikehara, Y., Akimitsu, N., Arimitsu, N., Hamamoto, H., Sekimizu, K., Suzuki, H., Igarashi, I., 2007. Glycophorin A-knockout mice, which lost sialoglycoproteins from the red blood cell membrane, are resistant to lethal infection of *Babesia rodhaini*. Vet. Parasitol. 148, 93–101.

Terkawi, M.A., Jia, H., Zhou, J., Lee, E., Igarashi, I., Fujisaki, K., Nishikawa, Y., Xuan, X., 2007. *Babesia gibsoni* ribosomal phosphoprotein P0 induces cross-protective immunity against *B. microti* infection in mice. Vaccine 25, 2027–2035.

Theiss, A.L, Idell, R.D., Srinivasan, S., Klapproth, J.-M., Jones, D.P., Merlin, D., Sitaraman, S.V., 2007. Prohibitin protects against stress in intestinal epithelial cells. FASEB J. 21, 197–206.

Untalan, P.M., Guerrero, F.D., Haines, L.R., Pearson, T.W., 2005. Proteome analysis of abundantly expressed proteins from unfed larvae of the cattle tick, *Boophilus microplus*. Insect Biochem. Mol. Biol. 35, 141–151.

Vlachou, D., Schlegelmilch, T., Christophides, G.K., Kafatos, F.C., 2005. Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. Curr. Biol. 15, 1185–1195.

Wang, H., Nuttall, P.A., 1999. Immunoglobulin-binding proteins in ticks: new target for vaccine development against a blood-feeding parasite. Cell. Mol. Life Sci. 56, 286–295.

Wasmuth, J.D., Blaxter, M.L., 2004. prot4EST: translating expressed sequence tags from neglected genomes. BMC Bioinformatics 5, 187.

Willadsen, P., 2004. Anti-tick vaccines. Parasitology 129, S367–S387.

Wilson, I.B.H., 2002. Glycosylation of proteins in plants and invertebrates. Curr. Opin. Struct. Biol. 12, 569–577.

Wilson, C.M., High, S., 2007. Ribophorin I acts as a substrate-specific facilitator of *N*-glycosylation. J. Cell Sci. 120, 648–657.

Yacoub, A., Kelley, M.R., Deutsch, W.A., 1996. *Drosophila* ribosomal protein P0 contains apurinic/apyrimidinic endonuclease activity. Nucleic Acids Res. 24, 4298–4303.

Zieler, H., Dvorak, J.A., 2000. Invasion *in vitro* of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. Proc. Natl. Acad. Sci. U.S.A. 97, 11516–11521.